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# An enzyme-linked immunosorbent assay for host cell protein contaminants in recombinant PEGylated staphylokinase mutant SY161

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**Abstract**

Staphylokinase, a bacterially-derived protein which functions as a plasminogen activator, has potential utility as a human therapeutic for thrombotic disorders. A recombinant version of this protein, SY161, contains 13 amino acid substitutions designed to decrease immunogenicity, and has been covalently modified by crosslinking a 5 kDa polyethyleneglycol (PEG) group to the N-terminal region to prolong the drug circulating half-life. The recombinant PEG-modified SY161 staphylokinase is currently in phase II clinical trials as a treatment for acute myocardial infarction. We have developed a sensitive product specific host cell protein (HCP) assay in the ELISA format to monitor in process host-derived contaminant clearance and final drug product purity. The assay is based upon use of goat polyclonal antibodies raised against *E. coli* host strain cell proteins from a null cell line, extracted by the same manufacturing process used to produce SY161. The identification and clearance of HCP contaminants was confirmed during drug product production using SDS-PAGE and Western blotting utilizing the same polyclonal HCP antibodies. The assay is specific for *E. coli* host cell strain proteins with a useful detection range from 1 to 100 ng/ml, and is not affected by product level. The level of residual HCPs in the clinical product produced by our manufacturing process was determined to be less than 1 ng/ml at a product concentration of 1 mg/ml. 

*Keywords:* Staphylokinase; *E. coli* host cell proteins; ELISA

## 1. Introduction

Recombinant protein pharmaceuticals produced as human therapeutics must contain levels of host and process-derived contaminants within safety limits established by international regulatory agencies [1,2]. Contaminants of particular

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importance in recombinant protein preparations include DNA, endotoxin and host cell-derived proteins. Although standardized quantitative methods are available for monitoring both DNA and endotoxin contamination, no generic test method is commercially available which is suitable to determine complex host cell-derived protein content in a process-specific context [3–5]. An accurate quantitative HCP contaminant determination is critical for establishing product release specifications, and can be useful in developing or modifying manufacturing processes, and for use in purification process validation [6–8].

Antibodies raised against host cell-derived proteins can be used in ELISA-type multi-well assay formats to provide quantitative determinations of HCP impurity levels in recombinant products, complementing other HCP contaminant identification methods such as SDS-PAGE, western blotting and HPLC [9–14]. This type of immunological detection method is possible using polyclonal antibodies prepared against strain-specific host cell proteins produced from the product purification process utilizing a null cell line (host cells containing the protein expression vector without the product gene sequence) [15]. Criteria that must be considered during development of such immunologic analysis include contaminant specificity, non-interference by product at high relative concentrations, and low detection limits. Detection of residual HCP levels in the range of 1–100 ppm, depending upon product type and dosing, are the currently expected assay limits for recombinant protein products [16,17]. It is expected that ELISA-type HCP test results will demonstrate consistency with the results from silver stained SDS-PAGE and chromatographic analyses of product purity [18–20].

Staphylokinase (Sak), a 15.3 kDa protein secreted by certain strains of *Staphylococcus aureus*, is one of a number of new thrombolytic agents which are currently being studied as human therapeutics for diseases which result in or stem from circulatory system blockage [21]. It forms a complex with human plasminogen, converting it to plasmin through an autoproteolytic event, which in turn activates other plasminogen molecules, resulting in the degradation of fibrin clots and the restoration of blood flow in occluded vessels [22]. The two most common plasminogen activators currently in clinical usage are tissue plasminogen activator (TPA) and streptokinase. Staphylokinase has potential advantages as a thrombolytic agent over current therapies, allowing simpler dosing regimens and greater clot-specificity, with concomitantly lower instances of abnormal bleeding events [30]. Two major disadvantages to the therapeutic use of Sak are the immunogenicity of the bacterial-derived protein in humans, which precludes repeat dosage, and a short circulating half-life in vivo. The recombinant Sak variant (SY161), with 13 amino acid substitutions, is designed to exhibit decreased immunogenicity and is currently in phase II clinical trials as a treatment for acute myocardial infarction [23–25]. The protein is expressed as a soluble product in *E. coli* and has been purified for use in clinical trials at large scale using a multi-step chromatographic process [26]. SY161 is PEGylated at a cysteine residue in the amino terminal region of the protein during the final stages of protein processing. This increase in the molecular hydrodynamic radius regulates clearance of circulating drug [27,28].

We have developed a highly sensitive and specific ELISA-based assay for strain-specific host cell-derived *E. coli* protein contaminants in SY161 product produced by our manufacturing process. It is based upon use of goat anti-HCP polyclonal antibodies derived from HCP isolated from null cells using our manufacturing process. These antibodies have been used as sensitive and specific probes in western blots to identify process-related contaminant proteins, and in ELISA format to quantitate process HCP residuals in clinical product. The levels of HCP specificity, sensitivity and broad range of contaminant HCP reactivity indicate that this assay is suitable and qualifiable for use in product release testing for phase III and later clinical materials, as well as for purification process validation.

## 2. Experimental

### 2.1. Apparatus

The Spectra Max Plus plate reader was obtained from Molecular Devices (Sunnyvale, CA). The AKTA Explorer chromatography system and Hoefer SemiPhor semi-dry transfer apparatus were obtained from Amersham Phamacia Biotech (Piscataway, NJ). The 3D Rotator was obtained from Lab Line (Melrose Park, IL). The model M-110Y Microfluidizer processor was from Microfluidics (Newton, MA). The GS-710 calibrated imaging densitometer was obtained from Bio-Rad (Hercules, CA). PVDF membrane (0.2 μm) was purchased from Invitrogen (Carlsbad, CA). CoStar 96-well assay plates (high binding capacity) were purchased from Corning (Corning, NY)

### 2.2. Reagents

CNBr-activated Sepharose 4B and NAP10 columns were obtained from Amersham Phamacia Biotech (Piscataway, NJ). Activated alkaline phosphatase (AP) was from Life Technologies (Rockville, MD). Western Blue® stabilized substrate for alkaline phosphatase was from Promega (Madison, WI). Alkaline phosphatase substrate kits and non-fat dry milk were from Bio-Rad (Hercules, CA). Micro BCA kits were obtained from Pierce (Rockford, IL). Celpure P1000 was purchased from Advanced Minerals (Santa Barbara, CA). Polyethyleneimine (PEI) was from Spectrum (Gardena, CA). Sodium phosphate, sodium chloride, Tween 20, EDTA, Tris base, acetic acid, sodium acetate, sodium carbonate, sodium bicarbonate, sodium borohydride, and glycine were from J.T. Baker (Phillipsburg, NJ). Bovine serum albumin (BSA), rabbit anti-goat IgG-AP conjugate, triethanolamine and boric acid were from Sigma (St. Louis, MO). Reagent water was obtained from a Millipore Milli-Q water-purification system (Bedford, MA). All other chemicals were analytical reagent grade unless otherwise indicated.

### 2.3. Working solutions

All working solutions were sterile filtered by utilizing a sterile, low extractable membrane, 0.22 μm, mounted on a disposable polystyrene bottle from Corning (Corning, NY). These solutions were stored in the refrigerator and were stable for at least 1 month unless indicated otherwise. The coating solution for the microplate was 1:1000 diluted affinity-purified polyclonal antibody against host cell protein (HCP) in 0.1 M sodium carbonate/bicarbonate buffer, pH 9.8, containing 0.05% sodium azide. Washing solution for the Western blot and microtiter plate was Tris-buffered saline (TBS: 8 g NaCl, 0.2 g KCl and 3 g Tris base in 1 l, pH adjusted to 7.4 with HCl) containing 0.02% Tween 20. Blocking solution for the Western blot and microtiter plate was either 5% non-fat dry milk (for Western blot) or 1% BSA (for microplate) in borate-buffered saline (170 mM boric acid and 120 mM sodium chloride, pH adjusted to 8.5 by NaOH) containing 0.05% sodium azide, 0.02% Tween 20 and 1 mM EDTA. The binding solution either for antigen (HCP) or antibody (alkaline phosphatase-antibody conjugate) was phosphate buffered saline (PBS: 10 mM sodium phosphate, 140 mM sodium chloride, pH 6.8) containing 0.05% sodium azide.

### 2.4. Preparation of process-specific host cell proteins (HCPs)

A mock fermentation run using the *E. coli* host cells containing the production plasmid but not the SY161 gene was performed to obtain the most probable HCPs present at different stages of purification of SY161. Frozen cell paste (0.562 kg) was suspended in 3.77 l of 50 mM sodium phosphate buffer, pH 9.5 containing 5 mM EDTA. The cells were lysed by passage through a microfluidizer at a pressure of approximately 13 000 psi. The lysate was adjusted to pH 7.5 using 10% phosphoric acid. A 5% PEI stock solution was added to the lysate to a final concentration of 0.2%. Celpure P1000 was then added to the flocculated lysate with stirring to a final concentration of 7% (w/v). The lysate/Celpure mixture was clarified by using a Komline–Sanderson Model

177 laboratory filter press. The clarified lysate was then diafiltered into PBS, pH 7.0 and diluted to a final protein concentration of approximately 1 mg/ml (determined by BCA method). The HCP pool at this stage was then used as the standard for the ELISA and for immunizations to produce specific antisera. This pool was also used to make the HCP-Sepharose 4B for further purification of resulting antisera.

### 2.5. Preparation of polyclonal antibodies against HCP

Antibodies against the HCPs were raised in goats according to established procedures. Each goat was immunized subcutaneously with 2 mg HCPs in complete Freund's adjuvant for the initial immunization. Subsequent injections (1 mg) were made every 2 weeks for the first 2 months and every 3 weeks in the next 2 months in incomplete Freund's adjuvant. Sera were collected and pooled. Affinity-purified anti-HCP antibodies were obtained by purifying antisera, diluted 3-fold with PBS, on a column containing HCPs coupled to CNBr-Activated Sepharose 4B. After thoroughly washing the column with PBS (10 column volume), the antibodies were eluted with 0.1 M sodium acetate buffer, pH 3.0 containing 0.1 M NaCl. The eluted fractions containing affinity-purified antibodies were immediately neutralized to pH 7.0 by adding 2 M Tris base solution. The antibody preparation was then buffer exchanged into PBS by diafiltration in an Amicon stirred cell with a 10 K molecular cutoff membrane. This preparation was then concentrated to approximately 3 mg/ml (determined by $A_{280}/1.44 = 1$ mg IgG/ml), sodium azide was added to a final concentration of 0.05%, and stored in aliquots at $-70$ °C. This affinity-purified antibody preparation was used for Western blotting, and as the capture antibody and detection antibody in the ELISA assay.

### 2.6. Preparation of alkaline phosphatase-antibody conjugate

Activated alkaline phosphatase (AP) was coupled to affinity-purified antibody according to the vendor's instructions. Coupled AP–Ab conjugate was fractionated on a NAP-10 column and the product was dialyzed against 2 l of triethanolamine buffer, pH 7.6 overnight at 4–10 °C. The AP–Ab was then stabilized by adding BSA to a final concentration of 10 mg/ml, aliquoted, shock frozen, and stored at $-70$ °C. This AP–Ab conjugate was diluted 1:750 with PBS as the detection antibody in the ELISA.

### 2.7. SDS-PAGE and Western blot analysis

The standard HCPs, SY161 and intermediate samples from different stages of purification ('in-process' samples) were analyzed by SDS-PAGE using 16% polyacrylamide mini gels (Novex, San Diego, CA) under a constant current of 35 mA per gel for approximately 40 min.

Western blots were performed according to Sambrook et al. [29]. The proteins were transferred from the SDS-PAGE gels onto a PVDF membrane using a semi-dry transfer apparatus at a constant current of 80 mA for 1 h. After transfer, the PVDF membranes were either stained with Coomassie Blue to show the transferred protein bands or treated to block excess protein binding sites with blocking buffer. The blocked PVDF membranes were then incubated either with antisera or with affinity-purified antibodies overnight. After washing, the membranes were further incubated with rabbit anti-goat IgG-AP conjugate for 2 h, and then the bound antibody was detected by exposing the membrane to substrate solution (Western Blue® stabilized substrate for AP) for 10 min as described in the vendor's instructions.

### 2.8. ELISA procedure

The ELISA procedure was performed as follows: polystyrene 96-well assay plates (CoStar, high binding capacity plate) were coated overnight at 4 °C with 50 μl/well of affinity purified anti-HCPs (3 μg/ml) in carbonate buffer, pH 9.8. The plates were washed in TBS, pH 7.4, containing 0.02% Tween 20 (TTBS). Excess binding sites were blocked for 1 h with 300 μl/well of borate buffered saline, pH 8.5, containing 1%

BSA, 0.05% sodium azide, 0.02% Tween 20 and 1 mM EDTA (blocking buffer). The plates were washed, and 200 μl of the standards, controls and samples were added in triplicate to the appropriate wells, and the plates were incubated for 2 h at room temperature with agitation on a plate shaker. Calibration standards, controls and the samples were initially diluted in PBS pH 6.8, with 0.05% sodium azide (PBSN) containing 0.25% BSA. The plates were washed again and 50 μl of AP-labeled anti HCP antibodies in PBSN were incubated in each well for 2 h at room temperature with agitation. After another wash step, 100 μl of enzyme substrate solution, prepared according to the manufacturer's instructions, was added to each well and the plates were incubated for 45–60 min at room temperature with agitation. Absorbance was measured at 405 nm on a Spectra Max Plus plate reader. The concentration of HCPs in the test samples was interpolated from the calibration curve obtained from HCP standards using both an unweighted linear and polynomial curve fit.

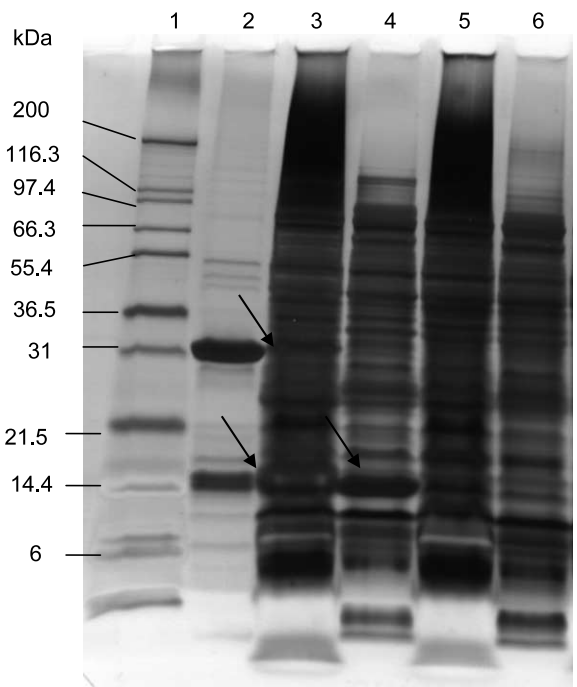


Fig. 1. Silver stain of SDS-PAGE for lysate and filtrate from production cell line and null cell line. Lane 1, protein markers; Lane 2, reference standard for product monomer ( ~ 15 kDa) and dimer ( ~ 30 kDa); Lane 3 and 4 are lysate and filtrate from production run. Arrow indicates the product band; Lane 5 and 6 are lysate and filtrate from null cell mock run.

### *2.9. Calculation of HCP content in SY161*

Samples of HCPs were assayed as a dilution series in the ELISA assay to ensure that antibody excess was established. HCP concentration of the samples was interpolated from the standard curve in units of ng HCP/ml and ppm content was calculated as ng HCPs/mg SY161. Samples that yielded a constant ppm value (plateau) with dilution were presumed to be in antibody excess for the detected HCPs. Values obtained from the triplicates were averaged and reported.

## 3. Results

### *3.1. Analysis of HCP standard and anti-HCP antibodies*

The HCPs obtained from the null cells of the production cell line mock run were over a wide range of molecular weights from larger than 100 kDa to smaller than 14 kDa. Fig. 1 shows the comparison of lysate and filtrate preparations from a normal production run and the mock run by SDS-PAGE with silver staining. Lanes 3 and 5 are the lysate, and lanes 4 and 6 are the filtrate. They show very similar visible band patterns, except no product bands (15 and 30 kDa) in lane 5 and 6, which are the samples from the null cell mock run. From this comparison, the HCP prepared from the null cell mock run (lane 6) was considered representative for the process. This HCP preparation was used for immunization of goats, as well as for the standard for Western blots and ELISA.

The antisera and affinity-purified polyclonal antibodies against HCPs were evaluated by Western blot to check the reactivity of the antibodies to the broad range of HCPs. Samples from different purification stages, final production bulk as well as the reference standard for HCP were loaded on the same blot. Fig. 2A shows the blot stained with

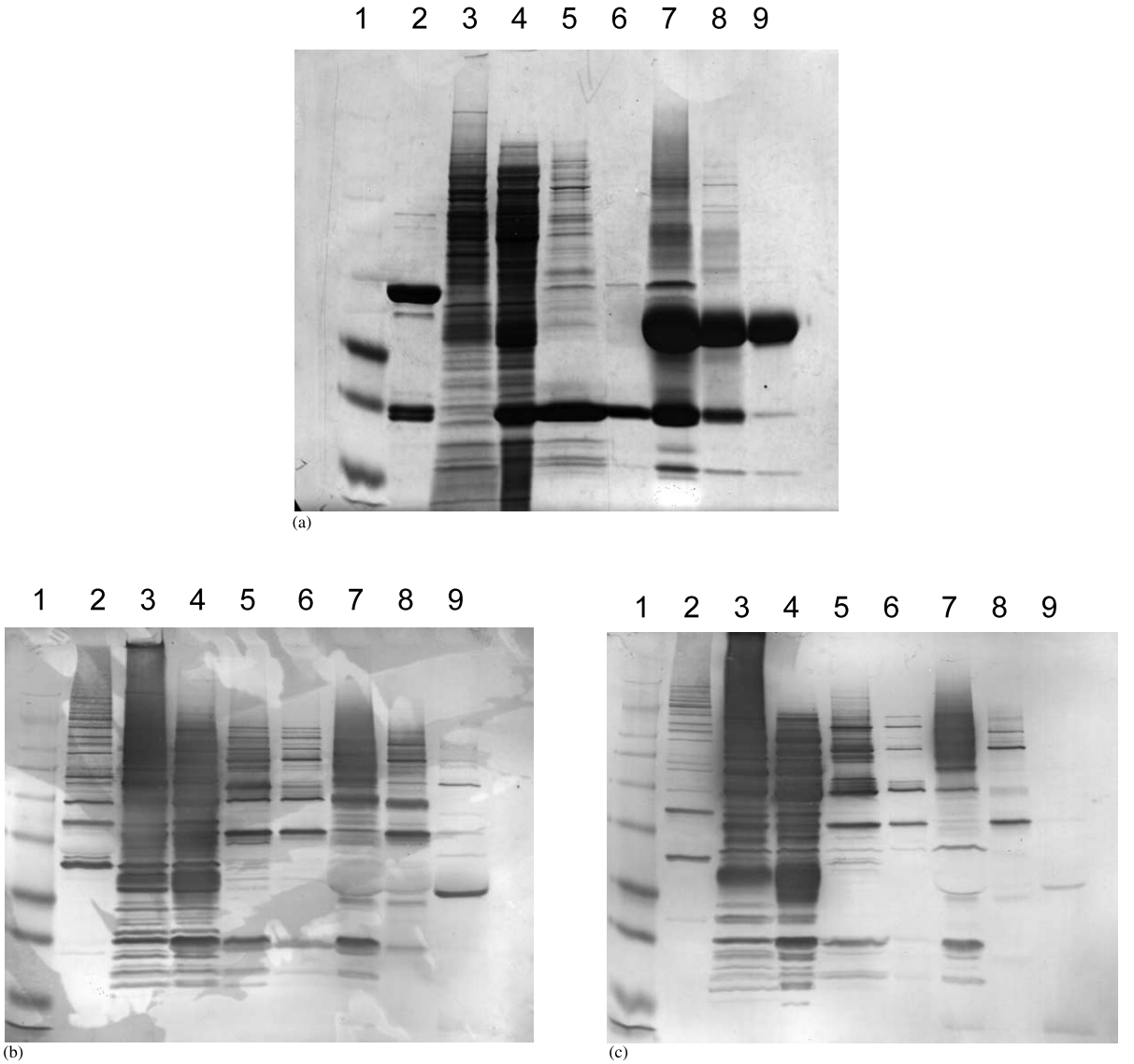


Fig. 2. Western blot analysis of in-process samples. Same samples were loaded on to three blots. Lane 1, protein markers; lane 2, SY161 reference standard (monomer: ~15 kDa, dimer: ~30 kDa); lane 3. HCPs reference standard; lane 4, lysate; lane 5, product fraction from purification step 1; lane 6, product fraction from purification step 2; lane 7, PEGylated product; lane 8, product fraction from purification step 4; and lane 9, final product. A is the blot stained by Coomassie blue to light up all protein bands. B and C are the same blot developed by antisera (2B) and affinity-purified HCP antibodies.

Coomassie blue to detect all the protein bands, including the SY161 monomer (15 kDa in lane 2, 4, 5 6, 7 and 8) and dimer (30 kDa in lane 2 and 7), as well as the PEGylated SY161 (20 kDa in lane 7, 8 and 9). Fig. 2B and C are the same blot developed with antisera and affinity-purified antibodies. Blots 2B and 2C show the same protein patterns as they did in 2A, except without the above-mentioned product related bands. This result indicates that the antisera and the affinity-purified HCP antibodies had no cross-reaction to any product related protein bands. Blots 2B and

Table 1
The HCP standard curve readings and their statistical correlation

| HCP concentration (ng/ml) | Background corrected absorbance ($A_{405\ nm}$) | | | Mean | S.D.[a] | CV | Back calculation (ng/ml) | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | 1[b] | 2[c] |
| 1 | 0.0159 | 0.0145 | 0.0152 | 0.015 | 0.001 | 4.61 | −1.04 | 1.05 |
| 4 | 0.0648 | 0.0712 | 0.0663 | 0.067 | 0.003 | 4.96 | 2.66 | 4.06 |
| 10 | 0.1728 | 0.1708 | 0.1772 | 0.174 | 0.003 | 1.89 | 10.19 | 10.28 |
| 25 | 0.3964 | 0.4097 | 0.3942 | 0.400 | 0.008 | 2.10 | 26.26 | 24.04 |
| 50 | 0.7726 | 0.7996 | 0.8166 | 0.796 | 0.022 | 2.79 | 54.35 | 49.97 |
| 100 | 1.3673 | 1.3969 | 1.4502 | 1.405 | 0.042 | 2.99 | 97.51 | 96.38 |

[a] S.D. of the mean.
[b] Back calculation according to a linear fit.
[c] Back calculation according to a polynomial fit.

2C exhibited very similar images. The only difference was that the blot 2C showed sharper protein bands and lower background than blot 2B. This indicated that affinity-purification of the antibody preparation eliminated some non-specific binding from the antisera. The lower background in blot 2C also made the faint, low molecular weight impurities (approximately 6 kDa) visible, which indicated necessary improvements in the purification process. In addition, the trend of HCP removal at each step of the purification was clearly demonstrated in these blots.

From the results obtained in SDS-PAGE and Western blots, the HCP reference standard and the affinity-purified antibodies were qualified for the further ELISA development in terms of the process representativity and specificity.

### 3.2. Data acceptance and evaluation

Unlabeled and AP-labeled affinity-purified anti-HCP antibodies were optimized for using in the antibody-sandwich ELISA to measure the HCPs. A standard curve was generated with HCPs in the range of 1–100 ng/ml. Triplicate samples were tested for different concentrations of HCP reference standard, the process intermediates and the final bulk to obtain the mean value of the assay and standard deviation (S.D.). There was no significant absorbance observed in the buffer blank (data not shown). Table 1 shows the background corrected readings at 405 nm from replicate serial dilutions of HCP reference standard performed by a single analyst on 1 day. The percent coefficient of the variation (%CV) for HCP concentrations from 1 to 100 ng/ml is below 5%. The mean data from these analyses were fit to either a linear or a polynomial (Fig. 3) curve, yielding $r^2$ values over 0.99 in both cases. Back-calculation of the mean standard data using the linear fit (Table 1) indicates that the HCP concentration determined in the ELISA are linearly correlated across a range from 10 to 100 ng/ml. The back-calculated mean standard data using the polynomial fit indicate accurate HCP concentration determinations

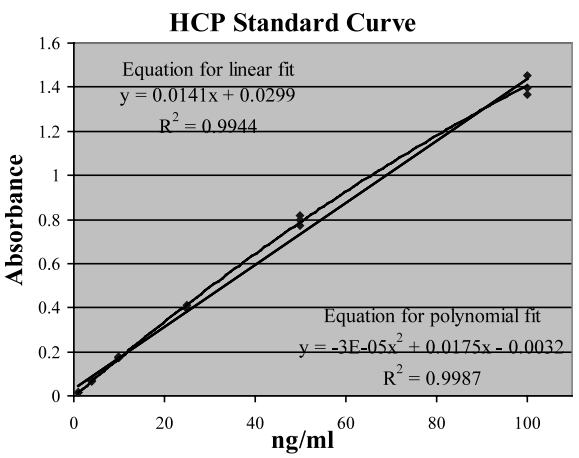


Fig. 3. HCP standard curves. The data from a concentration range from 1 to 100 ng HCPs/ml were fit to both linear and polynomial curve.

Table 2
Inter-assay reproducibility of the standards

| Expected HCP concentration (ng/ml) | HCP concentration back calculated (ng/ml) | | | Mean | S.D. | CV |
|---|---|---|---|---|---|---|
| | Exp. 1 | Exp. 2 | Exp. 3 | | | |
| 1 | 1.05 | 0.88 | 0.79 | 0.09 | 0.13 | 14.73 |
| 4 | 4.06 | 3.79 | 4.00 | 3.95 | 0.14 | 3.61 |
| 10 | 10.28 | 10.35 | 9.97 | 10.20 | 0.20 | 1.99 |
| 25 | 24.04 | 25.14 | 25.58 | 24.92 | 0.79 | 3.19 |
| 50 | 49.97 | 49.44 | 49.03 | 49.48 | 0.47 | 0.94 |
| 100 | 96.38 | 98.21 | 97.51 | 97.37 | 0.92 | 0.95 |

Table 3
Sensitivity of the HCP ELISA

| | Absorbance of buffer blank | Absorbance of 0.5 ng HCPs/ml | Absorbance of 1.0 ng HCPs/ml |
|---|---|---|---|
| *N* | 5 | 5 | 5 |
| Mean | 0.082 | 0.089 | 0.097 |
| S.D. | 0.0008 | 0.014 | 0.0007 |
| %CV | 0.998 | 1.607 | 0.720 |
| Mean + 2S.D. | 0.0836 | | |
| Mean − 2S.D. | | 0.061 | 0.0956 |

across the full range of concentration tested, from 1 to 100 ng/ml (Table 1).

The inter-assay reproducibility of the standards is indicated in Table 2, where we have analyzed the back-calculated HCP concentrations for three serial dilutions of the HCP reference standard. The ELISA data in Table 2 were generated by two analysts on different days to assess inter-analyst and inter-day variation. The %CV for the data was below 5% across the concentration range from 4 to 100 ng/ml, rising to below 15% at the lowest concentration tested, 1 ng/ml.

### 3.3. Limit of detection/limit of quantitation

Five replicates each of the buffer blank, 0.5 and 1.0 ng HCPs/ml were assayed to establish the limit of detection of the standard in ELISA (Table 3). The mean and S.D. of the absorbance for the 1.0 ng HCPs/ml standard indicated that it was significantly different from the buffer blank. However, the absorbance for the 0.5 ng HCPs/ml standard fell out of the 95% confidence range (mean − 2S.D. was 0.061, overlapped with the buffer blank confidence range). This indicated a signal-to-noise restriction establishing the effective lower limit of the assay (1.0 ng HCPs/ml). Thus, 0.5 ng/ml standard was not included in the standard curve in routine assays.

An upper limit of 250 ng HCPs/ml was also challenged in the ELISA. It gave a mean absorbance at 405 nm 2.503, a S.D. of 0.90 and %CV of 3.61. Here absorbance over 2 is considered as out of detector linear range. Linearity of the curve in a multiple antigen immunoassay demonstrates the requisite condition of antibody excess [19]. Hence, the 250 ng/ml concentration was not included in the standard curve. The standard curve covered one log of the test sample concentration, with a correlation coefficient ($r^2$) over 0.99, generally satisfactory for quality control purposes. Thus, 10 and 100 ng standard were considered to be the limits of quantitation of this ELISA using a linear curve fit.

### 3.4. Quantitation of HCPs in final purified SY161

The standard curve was established under conditions without product present. The final purified product usually contained only very minor amounts of HCPs. A study was performed to demonstrate that the assay antibody could detect the HCPs in the final purified product and at various stages of the production process ('In-Process samples'). Table 4 shows eight final purified samples collected from different purification procedures tested for HCP content by this ELISA. Four of them (samples 5–8) showed the HCP concentration below 1 ng/ml. A spike experiment used a sample (0.5 mg product/ml containing 22.99 ng HCPs/ml) spiked with 5 or 30 ng HCPs, which showed HCP readings of 29.48 and 54.16 ng HCPs/ml respectively. The theoretical target values for these samples were 27.99 and 52.99 ng HCPs/ml. Thus, the spike was fully recovered. These results indicated that the interference from the product in this ELISA assay was negligible.

Table 5 shows the HCP reduction in the purification process. A nine log HCP reduction was observed for the entire process. Significant reductions were achieved at each step of the process. These results were in agreement with the results of direct blotting and Western blot (Fig. 3). When 'in-process' samples were analyzed by the HCP ELISA, plateau values were observed for the early purification steps. This indicated that antibody excess had been achieved for the determination of HCPs. As expected from the Western blot results, the HCP concentration decreased at later purification stages. No HCPs were detected in the final purified bulk (product concentration, 0.5 mg/ml), at an assay sensitivity of 1 ng HCPs/ml.

## 4. Discussion

We have demonstrated that the trace residue HCPs in our product can be detected below 1

Table 4
HCPs concentration in final purified SY161

| Samples | HCP concentration (ng/ml) | SY161 concentration (mg/ml) | HCP in product (ppm) |
|---|---|---|---|
| Sample 1 | 33.97 | 1.17 | 29.03 |
| Sample 2 | 3.97 | 1.19 | 3.34 |
| Sample 3 | 1.98 | 1.17 | 1.69 |
| Sample 4 | 28.27 | 0.88 | 32.13 |
| Sample 5 | $<1.0$[a] | 1.19 | $<1.0$ |
| Sample 6 | $<1.0$[a] | 1.01 | $<1.0$ |
| Sample 7 | $<1.0$[a] | 1.23 | $<1.0$ |
| Sample 8 | $<1.0$[a] | 1.72 | $<1.0$ |

[a] The lowest standard was 1.0 ng HCPs/ml.

Table 5
HCPs reduction in purification process

| Purification step | HCP concentration (ng/ml) | Volume (l) | Total HCP (g) | HCP reduction[a] (%) |
|---|---|---|---|---|
| Lysate | $43.19\times10^{6}$ | 49 | 2116 | |
| Filtrate | $17.89\times10^{6}$ | 53.7 | 960.7 | 54.6 |
| Step 1 | $7.24\times10^{6}$ | 69 | 499 | 48.1 |
| Step 2 | $2.17\times10^{3}$ | 76.5 | 0.166 | 99.97 |
| Step 3 | 98.7 | 215.3 | 0.021 | 87.3 |
| Step 4 | 118.6 | 15.7 | $1.8\times10^{-3}$ | 91.4 |
| Step 5 (final) | $<1.0$ | 50.1 | $<50\times10^{-6}$ | $>99.0$ |

[a] HCP reduction calculated based on: total HCP in step ($n$) divided by total HCP in step ($n-1$), then times 100.

ng/mg protein by the enzyme-linked immunosorbent assay described above. This method can be qualified to have the reproducibility and robustness demanded for recombinant protein drug product quality control. By SDS-PAGE and Western blot methods, the assay antibodies demonstrated a broad spectrum of reactivity with mock run proteins and with in-process HCPs (Fig. 2). In addition, plateau phenomena were observed in the HCP ELISA for in-process samples. This assay is valid for detection of potential HCPs in all stages of the SY161 recovery.

Process-specific host cell protein immunoassays have been reported for other recombinant polypeptide therapeutics, including somatotropin [10], interferon [9] and erythropoetin [14]. The assay developed to quantitate residue *E. coli* HCPs in somatotropin and CHO cell HCPs in erythropoetin both utilized the Threshold light-addressable potentiometric sensor (LAPS) system. This more complicated sandwich assay format utilizes both fluorescien and biotin-labeled anti-HCP antibodies in conjunction with antifluorescein-urease conjugate. Hydrolysis of added urea by the captured urease conjugate generates ammonia that alters the pH of the solution and changes the surface potential on the sensor, providing the measured signal. The detection range reported for the Threshold CHO HCP assay was 5–400 ng/ml and 2–160 ng/ml for the Threshold *E. coli* HCP assay. The residual *E. coli* HCP assay reported for interferon was developed in the traditional ELISA format that we have used for PEGylated staphylokinase. In the case of interferon, the process-specific HCP assay showed significant product-related signal augmentation that required product level control to compensate for enhanced immunoreactivity. Their data clearly indicates the necessity for the development of product and process-specific residual HCP assays. In our study with PEGylated recombinant staphylokinase we detected no immunoreactivity alterations related to levels of product pre or post PEGylation. All three immunoassays discussed here found a best fit for their data across the full range of test data using a polynomial equation, as we have found with our data.

For the ELISA described here, some assay reagents, such as the affinity-purified antibodies for capture and the alkaline phosphatase labeled affinity-purified antibodies for detection, should be prepared by the test laboratory. Careful qualification of these reagents and validation of the method are absolutely necessary to eliminate the variation and interference caused by the reagent preparation and analysts. The HCP ELISA can provide accurate and precise quantitation of host cell protein impurities at ng/ml levels.

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